recognized synthetic peptides corresponding to these two regions (Atassi et al., 1996, *J. Prot. Chem.* 15, 691-700). Other experiments suggested that these two peptides may also contain T-cell epitopes (data not shown). Mice injected with low doses of BoNT/A H_c produced high titers, that were much higher than those elicited by the peptides. We observed the high titers after the third immunization. End-point antibody titers against H_c for mice injected with BoNT/A H_c are shown in **FIG. 9***c*. Control peptide produced little to no antibody to either peptide or BoNT/A H_c (**FIG. 9***d*). These data substantiate the fidelity of the synthetic peptides in vivo in mimicking the immunogenicity of the naturally processed BoNT/A.

[**0082**] Taken together, the results so far suggested that the epitopes detected by MAbs were good immunogens and, therefore, the peptides were used as candidates in a preliminary vaccine trial. Mice were immunized with a single peptide, combination of peptides, or BoNT/A H_c, and challenged 3 weeks after the final immunization with BoNT/A (Table 5). The highest dose was most effective in eliciting high antibody titers for all peptides (data not shown). All mice that were vaccinated with 2 or 10 μg of peptide and challenged with 10 LD_{50} of BoNT/A died. Mice immunized with peptide 1 were not protected against any lethal challenge doses. Vaccination with peptide 2 resulted in 40% survival when mice were given 10 or 40 μg and challenged with 5 or 10 LD_{50}, respectively. Slightly more protection was afforded when combination of peptide 1 and 2 were used in the vaccination protocol. This finding is consistent with the hypothesis that these two peptides may be a part of a single protective epitope on the Hc of BoNT/A. All mice vaccinated with BoNT/A H_c were completely protected against challenge. Mice that received control peptide, then challenged with 5 or 10 LD_{50} of BoNT/A were all killed. No noticeable effects on the outcome of these experiments were observed when peptides were conjugated to KLH, or administered with other adjuvants (data not shown).

TABLE 5

Vaccine potential of 25-mer designed peptides

Immunizing[a] agent	Dose[b]	Challenge[c] (μg/mouse)	Live/Total[d] (LD50)
Peptide 1	2	10	0/5
	10	10	0/5
	40	10	0/5
	10	5	0/5
Peptide 2	2	10	0/5
	10	10	0/5
	40	10	2/5
	10	5	2/5
Peptide 1 + 2	2	10	0/5
	10	10	0/5
	40	10	3/5
	10	5	3/5
BoNT/A Hc	2	1,000	5/5
Control Peptide	40	5	0/5

[a]Mice were vaccinated i.p. and boosted at 3, 6, and 9 weeks.
[b]Immunizing dose of each immunogen.
[c]Mice were challenged with BoNT/A 3 weeks after the final boost.
[d]Lethality was recorded 7 days after the challenge dose.

[**0083**] One of the goals of this study was to validate the predictive abilities of molecular modelling studies that used theoretically derived predictions of secondary structure and solvent accessibility of the residues to identify surfaces that interact with MAbs. We reasoned that careful attention to the kinetics of MAbs may permit us to combine the benefit of modelling with the high-affinity neutralizing MAbs to identify selected regions that may play a critical role in forming PPADS. To substantiate further this logic, low-affinity MAbs that recognize the peptides or BoNT/A H_c are being tested for their ability to protect mice against BoNT/A.

[**0084**] There is some evidence from this study and previously published data that significant discontinuity may exist in neutralizing epitopes within BoNT/A (Dertzbaugh and West, 1996, supra). In addition, epitope mapping of these MAbs by using a constrained-peptide display library supports the existence of discontinuous epitopes within BoNT/A (M. Segall and S. Bavari, unpublished data). Therefore, we believe a single, short-peptide vaccine may not be feasible for generating protective immunity against BoNT/A. However, because a single MAb with a very high affinity can block the lethality of BoNT/A, it might be possible to design a vaccine with a combination of two peptides.

[**0085**] Unlike BoNT/A H_c, the highest titers to the peptides were detected after the third boost. This may be due to unwanted degradation of the peptides before reaching receptive major histocompatibility complex class II molecules. In hope of increasing the immunogenicity of peptides, we are currently developing delivery methods to protect better the peptides from degradation in acidic compartments of antigen-presenting cells. To increase efficiency of forming complexes with major histocompatibility complex class II molecules, peptides are being engineered with various signal motifs that should more efficiently deliver the peptides to peptide-loading compartments of antigen-presenting cells.

[**0086**] In this targeted survey of two peptides, we have not yet explored details of the kinetics of association and dissociation for peptide and MAbs. However, the initial data suggest there is a correlation between the level of MAb binding to H_c of BoNT/A, and the effectiveness of the MAb peptide complex formation (D. Pless and S. Bavari, unpublished observations). A survey of amino acid substitutions will allow us to monitor the behavior of the two peptides and identify key amino-acid residues, particularly at solvent-exposed positions, that could alter binding to MAbs. This type of approach may further help to boost the efficacy of the peptides.

[**0087**] In conclusion, our study demonstrated that robust molecular modelling studies that predict secondary structure and locate highly solvent-exposed residues combined with very high-affinity neutralizing MAbs may be used to identify PPADs. This type of approach can be used as a viable alternative to the expensive and time-consuming methods of identifying neutralizing epitopes by synthesizing overwhelming numbers of long peptides.